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Cloning and developmental expression of the sucrose-phosphate-synthase gene from spinach

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Abstract. A 561-base-pair (bp) polymerase-chain-reaction (PCR) product of sucrose-phosphate synthase (SPS) was amplified using degenerate oligonucleotide primers corresponding to tryptic peptides of SPS (EC 2.4.1.14) from spinach (*Spinacia oleracea* L). Crucial to the primer specificity and the synthesis of the 561-bp product was the use of primer pools in which the number of degenerate primer species was limited. A full-length cDNA was subsequently obtained by screening a cDNA bacteriophage library with the 561-bp product of SPS and 5′ PCR-RACE (Rapid Amplification of cDNA Ends). The 3530-bp cDNA of SPS encoded for a 1056-amino-acid polypeptide of predicted molecular mass of 117 kDa. The deduced amino-acid sequence of spinach SPS showed regions of strong homology with SPS from maize (A.C. Worrell et al., 1991, Plant Cell **3**, 1121–1130); amino-acid identity was 54% over the entire protein. Western and Northern analyses of root, petiole and spinach leaf tissue showed that SPS was expressed in an organ-specific manner, being predominantly localized in the leaf. The accumulation of SPS protein and mRNA during leaf development coincided with the early rapid phase of leaf expansion and the apparent transition of the leaf from sink to source status. Levels of SPS mRNA and protein were reduced during the acclimation of leaves to low-irradiance conditions. Transfer of low-irradiance-adapted leaves to higher-irradiance conditions resulted in a gradual increase in SPS protein and mRNA. Diurnal changes in irradiance did not alter SPS protein or transcript levels, indicating that short-term regulation of SPS primarily involves a modulation of enzyme activity.

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Abbreviations: kbp = kilobase-pair; kDa = kilodalton; PCR = polymerase chain reaction; PCR-RACE = rapid amplification of cDNA ends; *rbcS*, *SAc*, *sps*1 = genes encoding the small subunit of Rubisco, actin, and SPS, respectively; Rubisco = ribulose-1,5-bisphosphate carboxylase-oxygenase; SPS = sucrose-phosphate synthase

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Introduction

Sucrose-phosphate-synthase (SPS) activity influences the rate of sucrose formation and the partitioning of photoassimilate between sucrose and starch (for review, see Stitt et al. 1987; Stitt and Quick 1989). Leaf SPS activity is regulated at two levels: (1) metabolic fine control; and (2) coarse control. Briefly, fine control of enzyme activity is exerted by metabolic effectors that instantaneously activate or inhibit catalysis. Coarse control refers to slower changes in the extractable activity of an enzyme caused by covalent modification or changes in the rate of enzyme synthesis and- or turnover. Coarse control is indicated by changes in SPS activity in response to light/dark transitions (Rufty et al. 1983; Huber et al. 1987, 1989; Stitt et al. 1988; Bruneau et al. 1991), source-sink manipulations (Rufty and Huber 1983; Rufty et al. 1984; Stitt et al. 1990), changes in development (Giaquinta 1978; Silvius et al. 1978; Huber and Israel 1982; Huber et al. 1987; Bruneau et al. 1991), and adaptation to low temperature (Guy et al. 1992).

Studies of the expression and regulation of SPS had long been limited to the measurement of enzyme activity in relatively crude extracts. Recently, SPS has been purified to near homogeneity from spinach (Salvucci et al. 1990) and maize leaves (Bruneau et al. 1991). The purification of the SPS protein facilitated immunochemical examination of enzyme accumulation in maize (Bruneau et al. 1991) and spinach (Walker and Huber 1989b). A further advancement was the isolation of a complementary DNA (cDNA) sequence of the maize SPS gene (Worrell et al. 1991). When the maize cDNA was expressed in transgenic tomatoes, total SPS activity was increased, resulting in a reduction of leaf starch and increase of sucrose.

These results provide strong evidence for the involvement of SPS in the regulation of photosynthate partitioning.

The objectives of the present study were to isolate a cDNA of the SPS gene from spinach leaves and to initiate studies of SPS gene expression. A relatively rapid strategy for cloning a full-length cDNA from spinach is detailed that utilizes a combination of the polymerase chain reaction (PCR), 5′ PCR-RACE (rapid amplification of cDNA ends), and conventional screening of a cDNA bacteriophage library. The cDNA of spinach SPS, *sps1*[1], was used to examine organ-specific, developmental and environmental factors affecting SPS gene expression. The expression of two well-characterized gene families, ribulose-1,5-bisphosphate carboxylase-oxygenase (Rubisco) small subunit (*rbcS*) and actin (*SAc*) were also examined as a basis for comparison with SPS.

Materials and methods[2]

Plant growth. Field-grown spinach (*Spinacea oleracea* L. cv. Melody) was planted under commercial growing conditions (approx. 68 kg N/ha). Unless stated otherwise, plants were sampled between 10 a.m. and 1 p.m.. For chamber-grown spinach, seeds were germinated and maintained in Conviron E15 environmental chambers under a 9 h/15 h day/night regime (irradiance 300–400 $\mu mol \cdot m^{-2} \cdot s^{-1}$, incandescent plus fluorescent bulbs) with an average day/night temperature of 21°C/15°C. Studies were conducted on spinach that was approx. two months old.

Synthesis of first-strand cDNA and PCR amplification. Tryptic peptides were obtained from purified SPS protein and amino-acid sequences determined (data not shown). Pools of oligonucleotide primers encoding portions of two SPS tryptic peptides, T28 and T31, were synthesized with the number of different species in each pool being minimized (Table 1). In brief, amino acids requiring four or six codons were avoided in the two consecutive codons of the 3′ terminus. A low level of complexity was achieved by incorporating dIMP (Ohtsuka et al. 1985) at three or four base degeneracies or the most stable deoxynucleotide (Martin and Castro 1985; Aarts et al. 1991) at two base degeneracies. These concepts were applied to all possible coding sequences except at the 3′ end of each primer pool where a perfect match is a prerequisite for elongation. To insure a perfect match within a primer pool at the 3′ terminus, pairs of primer pools were synthesized differing only by a single nucleotide at the degeneracy nearest the 3′ terminus (see Table 1, underlined nucleotides). Once these concepts were applied, primer pools were examined for primer-dimer formation and self-complementary duplexes using the OLIGO DNA Amplification Program (Rychlik and Rhoads 1989). Where indicated, 3′-terminus nucleotides were eliminated until primer-dimer formation and self-complementary duplexes were negligible.

For synthesis of first-strand cDNA, cesium-trifluoroacetate-purified (Pharmacia-LKB, Piscataway, N.J., USA) leaf RNA was isolated from field-grown spinach (cv. Melody) as described by Okayama et al. (1987). Polyadenylated mRNA was isolated by two cycles of chromatography on oligo(dT) columns. A 100-ng sample of poly(A)mRNA was used as template for synthesis of first-strand cDNA. Following denaturation of RNA (65°C, 5 min), random hexamers were annealed to the RNA and first-strand cDNA was synthesized with Moloney murine leukemia-virus reverse transcriptase (37°C, 1 h) under conditions recommended by the manufacturer (Stratagene, La Jolla, Calif., USA). Ten microliters of first-strand-cDNA reaction mixture was added to a 100-µl PCR reaction which contained 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% (w/v) gelatin, 0.2 mM of each dNTP, and 100 pmol of each of the appropriate sense and antisense primers directed against SPS tryptic peptides, T28 and T31 (see Table 1). The reaction mixture was heated to 91°C for 5 min, cooled to 51°C, and 2.5 U Amplitaq (Perkin-Elmer Cetus, Norwalk, Conn., USA) was added. The reaction was then overlaid with mineral oil and extended at 72°C for 2 min. Amplification for cycles 2–5 was performed through a regime of 1 min template denaturation at 97°C, 2 min primer annealing at 51°C, and 2 min primer extension at 72°C. Amplification for cycles 6–35 was performed as for cycles 2–5 except the template was denatured at 91°C for 80 s. After the final cycle, the reaction was extended for 8 min. Following amplification, the PCR product was purified by the GENECLEAN protocol (BIO 101; La Jolla, Calif.), blunt-end-ligated into SK plasmid and sequenced as described (Klein and Salvucci 1992).

Synthesis and screening of a spinach cDNA library. To obtain a full-length cDNA of spinach SPS, a Lambda-ZAPII cDNA library was synthesized and screened with the SPS-PCR product. To reduce mRNA secondary structure during synthesis of first-strand cDNA, 5 µg of poly(A)mRNA (total volume, 20 µL) was heated to (65°C, 5 min) and cooled to room temperature. Two microliters of 100 mM methylmercuric (II) hydroxide was added to the RNA solution and incubated for 1 min followed by the addition of 4 µL of 700 mM β-mercaptoethanol. After an additional 5 min, RNA was used for synthesis of first-strand cDNA. First-strand cDNA was primed with random hexamers and oligo(dT). A Lambda-ZAPII cDNA library was subsequently constructed as described in the Stratagene Lambda-ZapII cDNA Instruction manual. Approximately $1 \cdot 10^5$ primary plaques were screened with randomly labeled 561-base-pair (bp) PCR product of SPS under conditions recommended by the manufacturer (Stratagene). After four rounds of plaque purification, 10 potential positive plaques were identified. Partial sequence analysis of all SPS clones and the complete nucleotide sequences (coding and noncoding strands) of the three longest cDNAs (approx. 2.7 to 3.0 kbp in length) was obtained.

The technique of PCR-RACE (Rapid Amplification of cDNA Ends) was used to obtain the 5′ end of the SPS cDNA essentially as described by the manufacturer (GIBCO-BRL, Gaithersburg, Md., USA) except 100 ng of poly(A)mRNA was substituted for total RNA as template. The gene-specific (antisense) primer used to prime synthesis of first-strand SPS cDNA was 5′-TTCACCATAACTCCAATCTACACC (see Fig. 2, position 803–780 bp). Amplification by PCR used the 5′ anchor primer (supplied by GIBCO-BRL) and a second antisense SPS-specific primer (5′GTAGCCTCTCTGCGACCTCTTTC) which annealed to an internal, nested site (Fig. 2, position 454–432 bp) within the SPS cDNA. Amplification conditions were as described above except for an annealing temperature of 57°C. Following amplification, the PCR product was cloned and sequenced as described above.

Northern and Western blot analyses. Polyadenylated mRNA (0.5 µg per lane) was loaded on formaldehyde gels (Sambrook et al. 1989). The RNA was transferred to GeneScreen nylon membranes (DuPont-NEN, Wilmington, Del., USA), prehybridized, and then hybridized with radiolabeled antisense-RNA probes (58°C, 50% formamide). Conditions for synthesizing radiolabeled RNA and probe-hybridization conditions were as recommended (Stratagene, pBluescript II instruction manual). Blots were washed in 2 × SSC (1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate) plus 0.2% SDS (3 times, 10 min each at 58°C) and 0.1 × SSC plus 0.2% SDS (three times, 10 min each, 58°C). The Northern probe for *rbcS* (Rubisco small subunit) was a 743-bp antisense RNA of tobacco *rbcS* (Klein and Salvucci 1992); the probe for SPS was a 2700-bp antisense

[1] The GenBank accession number L04803, has been assigned to the nucleotide sequence of spinach SPS, sequence name, *sps1*.

[2] Mention of trademark, proprietary product, or vendor does not constitute a guarantee or warranty of the product by the U.S. Department of Agriculture and does not imply its approval to the exclusion of the other products or vendors that may also be suitable.

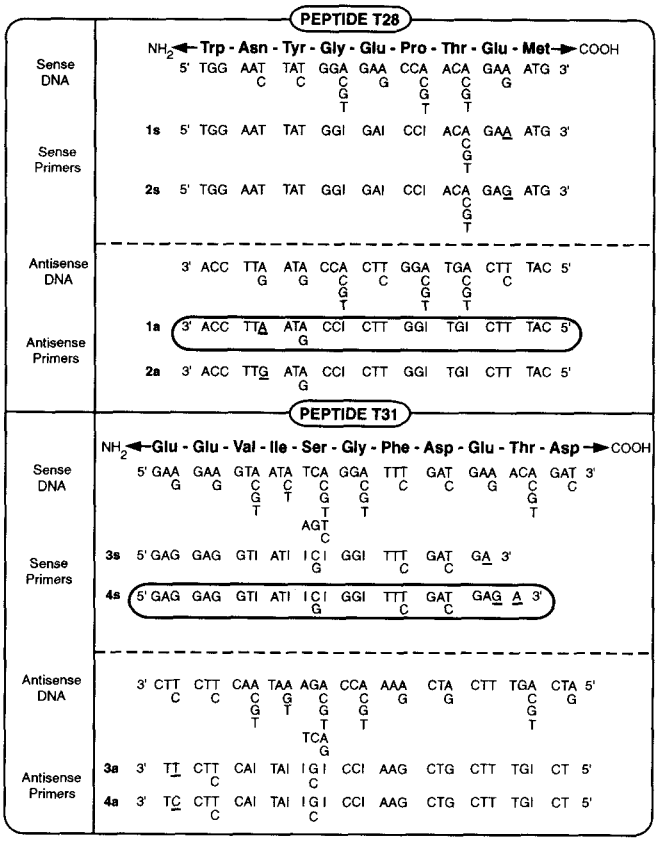
RNA from the open reading frame of spinach SPS; and the probe for actin was a 595-bp antisense RNA which contains a portion of the open reading frame of actin (*SAc3*) from soybean (kindly provided by R. Meagher, Department of Genetics University of Georgia, USA).

Western blots were conducted on spinach tissue extracts which were prepared as previously described (Crafts-Brandner et al. 1990). Samples of 20–40 μg of solubilized protein were separated on either 8% (for SPS detection) or 12% (for Rubisco detection) polyacrylamide minigels and transferred to nitrocellulose. Nitrocellulose filters were incubated overnight with either affinity-purified Rubisco holoenzyme antibodies or antibodies directed against a 21-kDa portion of spinach SPS expressed in *Escherichia coli* (data not shown). Polypeptides were visualized using an alkaline-phosphatase-conjugated secondary antibody system with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate as substrates.

Enzyme extraction and assay. Leaf tissue to be assayed for SPS enzyme activity was harvested and immediately placed in liquid N_2. The SPS activity was extracted by homogenizing 0.15 g of frozen leaf tissue (or 0.5 g of root or petiole tissue) in 1.5 mL of ice-cold grind buffer as previously detailed (Crafts-Brandner 1992). After centrifugation at 13000 · *g* for 30 s, 0.3 ml of the supernatant was desalted by passage through a column of Sephadex G50-300. Sucrose-phosphate synthase was assayed at 30°C by substrate-dependent formation of sucrose. Assays were initiated by the addition of extract (45 μL) to a reaction mixture (150 μL final volume) containing 50 mM Hepes-NaOH (pH 7.4), 15 mM $MgCl_2$, 1 mM EDTA, 7.5 mM fructose-6-phosphate, 7.5 mM uridine 5′-diphosphate glucose, 37.5 mM glucose-6-phosphate, and 10 mM KF. Assays were conducted in triplicate.

Miscellaneous. Protein was determined by the method of Bradford (1976) using bovine serum albumin as a standard. Amino-acid sequences were aligned with the FASTP software program (Lipman and Pearson 1985).

Table 1. Degenerate PCR primer pools designed according to the amino-acid sequence of SPS peptides T28 and T31[a]



[a] The combinations of primer pools which successfully directed the amplification of SPS target fragment are boxed

Results

Isolation of SPS cDNA, sequence determination and comparison. The partial amino-acid sequence of SPS enabled us to develop a molecular probe for the SPS gene using the polymerase chain reaction (PCR). Pools of deoxyinosine-containing oligonucleotide primers encoding part of two SPS tryptic peptides, T28 and T31, were synthesized with the number of different primer species in each pool minimized (Table 1). Using random-hexamer-primed first-strand cDNA as template, the combination of primer pools T28-1a and T31-4s directed the synthesis of a single 561-bp PCR product (Fig. 1, lane 7). Moreover, no detectable product was formed when primer mixtures in the opposite, apparently incorrect orientation were combined (lanes 2–5). The identity of the amplification product of lane 7 as encoding for SPS was confirmed by comparison of the deduced amino-acid sequence of the PCR product with tryptic and chymotryptic peptides from purified spinach SPS (data not shown). Several primer combinations including T28-2a and T31-4s (lane 9) yielded a detectable PCR product which probably reflects the relative tolerance of the system for base-pair mismatches near the 3′ terminus of the primer.

The PCR-amplified fragment of SPS was used to screen a random-hexamer-primed Lambda-ZAP II cDNA library for spinach leaf poly(A)mRNA. Ten partial clones were obtained with five clones having inserts greater than 2700 bp in length. Complete sequence analysis of the three longest SPS clones (and partial analysis of the remaining seven clones) showed that the sequence of all clones was identical in the coding region. Using two overlapping clones, a cDNA lacking only the 5′-untranslated nucleotides was assembled. The remaining 5′ sequence was obtained by PCR-RACE (for review see Frohman 1990). Using an SPS-specific antisense oligonucleotide for first-strand synthesis and a second nested gene-specific antisense primer for PCR amplification, a single 454-bp PCR product was obtained (data not shown). Southern-blot and sequence analysis confirmed the identity of the PCR product as encoding for the predicted 5′ portion of SPS including the 5′-untranslated region. In the region where the PCR-RACE product and the cloned cDNA of SPS have sequence in common, complete sequence identity was observed (Fig. 2, nucleotides 87–432). Given these overlapping clones, we were able to assemble the sequence of the SPS cDNA as shown in Fig. 2.

The SPS cDNA of 3530 bp in length contains a 5′-leader of 86 nucleotides and a 276-nucleotide 3′-untranslated region. The SPS cDNA encoded for a protein of 1056 amino-acid residues with a predicted molecular mass of 117 kDa which is in agreement with the apparent molecular mass of 120 kDa determined by electrophoret-

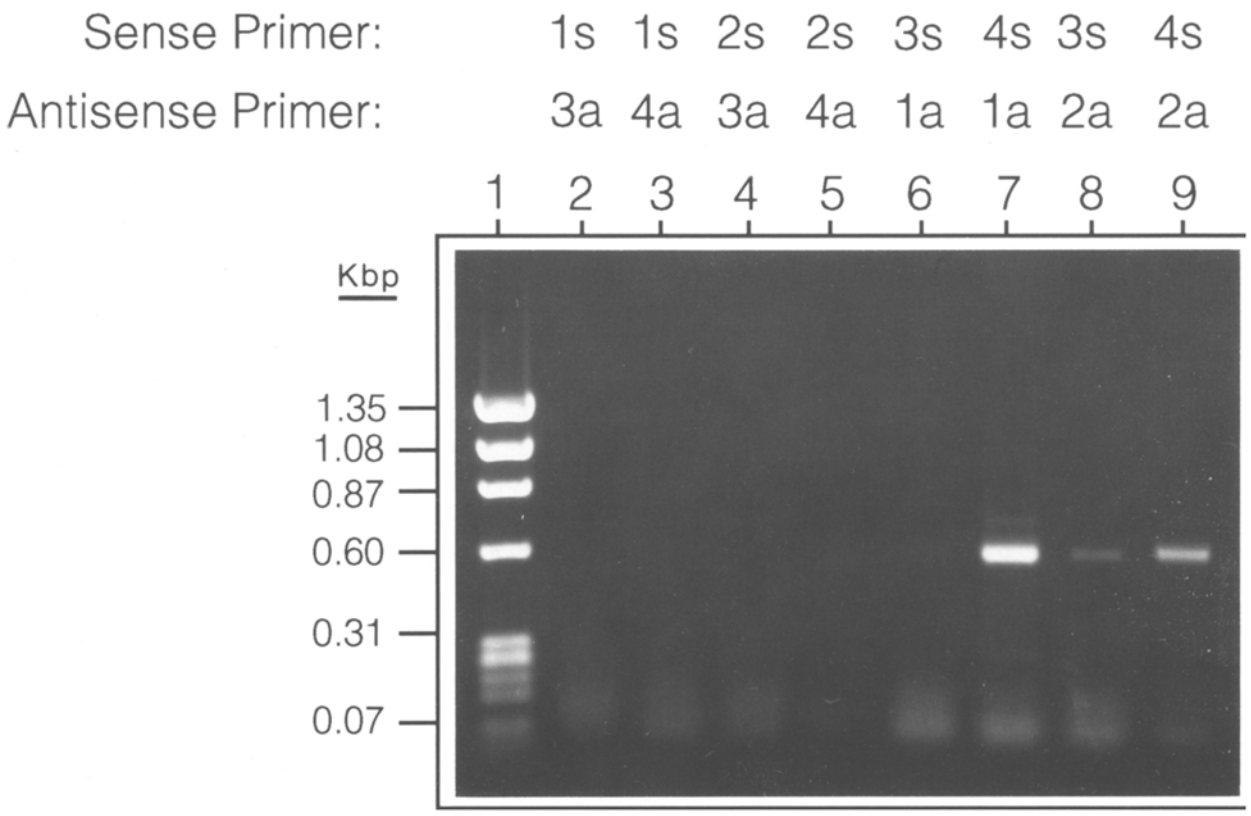


Fig. 1. Amplification products of PCR reactions directed by oligonucleotide primers corresponding to SPS tryptic peptides T28 and T31. The PCR primers were as shown in Table 1. *Lane 1* represents HindIII-digested Lambda DNA as molecular-size markers

ic mobility (Walker and Huber 1989a; Salvucci et al. 1990). Predictions of protein structure indicate that SPS should be soluble (data not shown). Comparison of the deduced amino-acid sequences of SPS from maize (Worrell et al. 1991) with spinach showed regions of high conservation, though divergent regions were also apparent (Fig. 3). Allowing for insertions and deletions to maximize alignment, an identity on the order of 54% over the entire maize and spinach enzyme was observed.

Northern and Western blot analyses of SPS. Northern and Western blot analyses were conducted on spinach tissues to examine several developmental and organ-specific parameters governing SPS gene expression. As a basis for comparison, the expression of genes encoding the Rubisco small subunit (*rbcS*) and actin (*SAc*) were concomitantly examined. The *rbcS* and the *SAc* gene families were examined since unique developmental and tissue-specific expression have been exhibited by these genes (Hightower and Meagher 1985; Kuhlemeier et al. 1987; McLean et al. 1990). Preliminary results indicated that SPS encoded a single species of mRNA of approx. 3.6 kb in length (see Fig. 4). Western analysis showed a single polypeptide of approximate molecular mass of 120 kDa. Examination of organ-specific expression revealed that the greatest proportions of SPS protein and transcripts were localized in green leaf tissue (Fig. 4). Sucrose-phosphate synthase protein and mRNA were detected in petioles, though the level of expression was approximately seven-fold lower than in leaves. The quantity of SPS protein and mRNA in root tissue was at the lower limit of Western- and Northern-blot sensitivity. The maximum extractable SPS enzyme activity of roots, petioles, and green leaves generally paralleled the accumulation of SPS protein as determined by Western blot analysis. Maximum extractable SPS activity of roots, petioles, and leaves (approx. 50% full expansion) was 7, 12, and 139 μmol sucrose · (g FW)$^{-1}$ · h^{-1}, respectively. In agreement with previously published results (for review see Kuhlemeier et al. 1987, 1989), Rubisco small- and large-subunit polypeptides and *rbcS* mRNA accumulated predominately in photosynthetic leaf tissue. The level of *rbcS* transcripts in petiole tissue was approximately 50-fold less than in green leaves while transcripts of *rbcS* were not detected in roots. In contrast, a greater proportion of actin (*SAc*) mRNA was detected in nonphotosynthetic tissue with the level of actin mRNA in root and petioles being several-fold greater than in the green leaf tissue. In addition, it should be noted that the exposure times of the Northern analyses of *sps*1, *rbcS*, and actin mRNA differ significantly and hence should be considered when comparing the absolute amounts of each transcript. The exposure times of SPS Northern analyses were consistently 10- to 15-times longer than that of *rbcS* Northern analyses, suggesting that SPS transcripts do not accumulate to nearly the level of *rbcS* in photosynthetic leaf tissue (assuming similar probe specific activity).

An examination of the accumulation of SPS protein

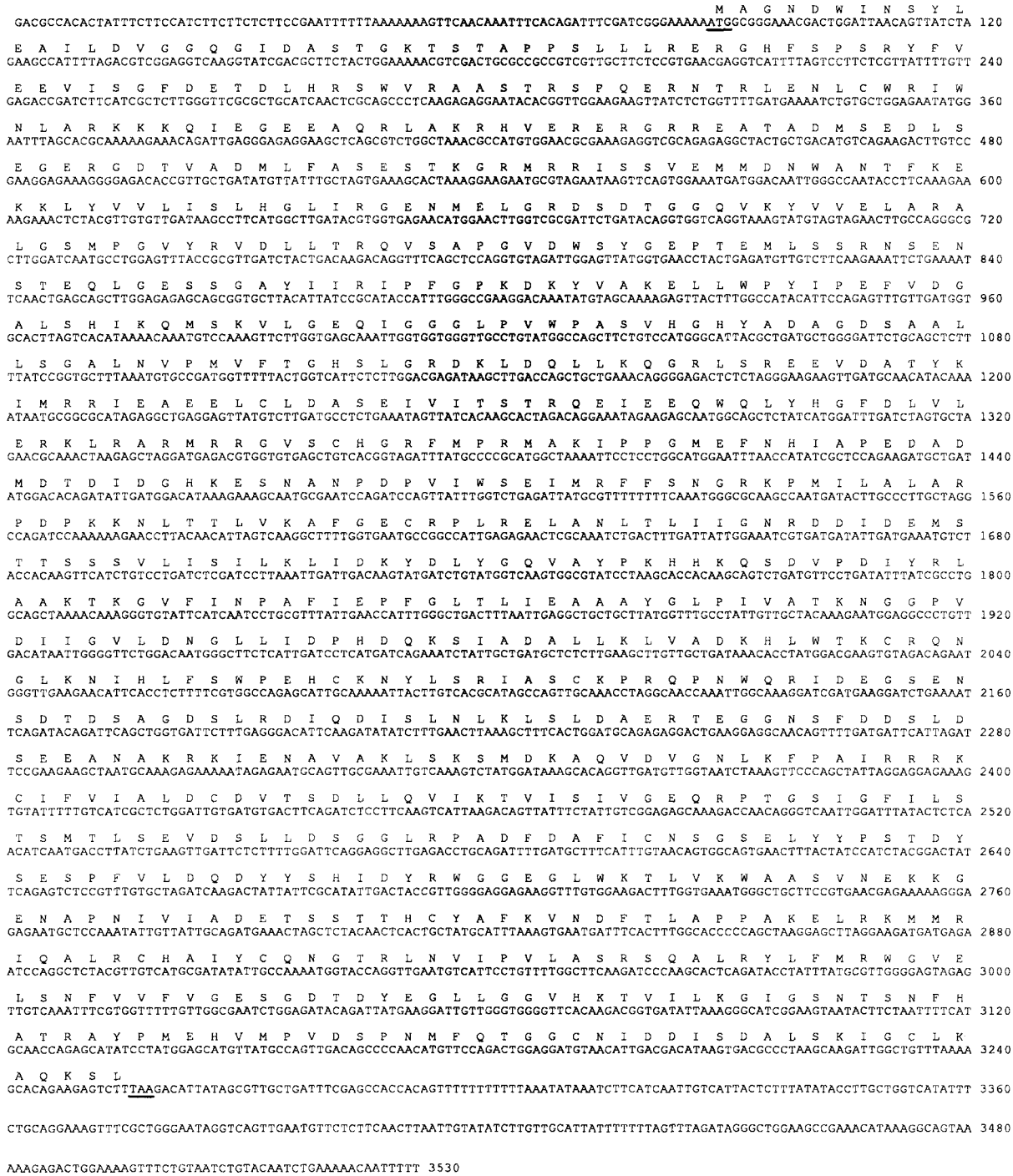


Fig. 2. Nucleotide and deduced amino-acid sequences of spinach SPS cDNA. The start and stop codons are *underlined*

and mRNA levels during spinach leaf development is shown in Fig. 5. A low level of SPS protein and mRNA was detected in small, partially unfolded leaves (leaf area 4 cm^2). Field-grown spinach leaves of this stage of development are yellow-green in color and hence, probably constitute sink tissue. Both SPS protein and mRNA accumulated during the period of rapid leaf expansion while no further increase was observed after leaves reached about 55 cm^2 in size (approx. 50%-full expansion). Activity of SPS enzyme increased as leaves expanded reaching an apparent maximum extractable activity of 139 μmol sucrose · $(g\ FW)^{-1} \cdot h^{-1}$ at about 50% of full leaf expansion (data not shown). Thereafter, SPS enzyme activity declined slightly (20%) as the leaf approached full expansion. The pattern of Rubisco protein and mRNA accumulation was similar to that of SPS although *rbcS*-transcript levels reached an apparent maximum at an earlier stage of leaf expansion (Fig. 5, lower panel). By comparison, actin-mRNA levels declined slightly as the leaf approached full expansion.

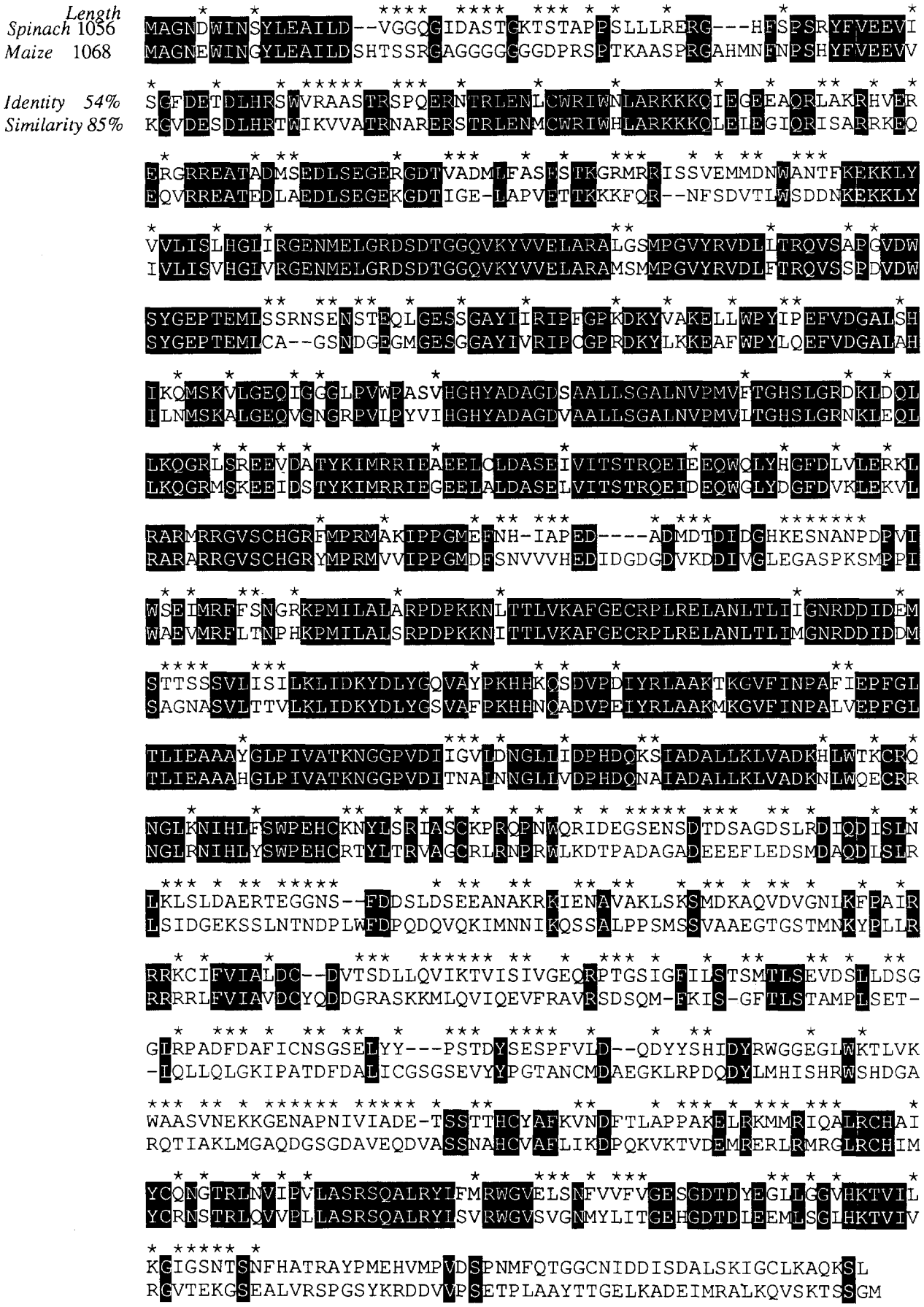


Fig. 3. Comparison of the deduced amino-acid sequences of SPS from maize (Worrell et al. 1991) and spinach. Identical amino-acids are indicated by black boxes and conservative amino-acid exchanges by *asterisks*. *Dashes* in the sequences have been introduced to obtain maximum alignment

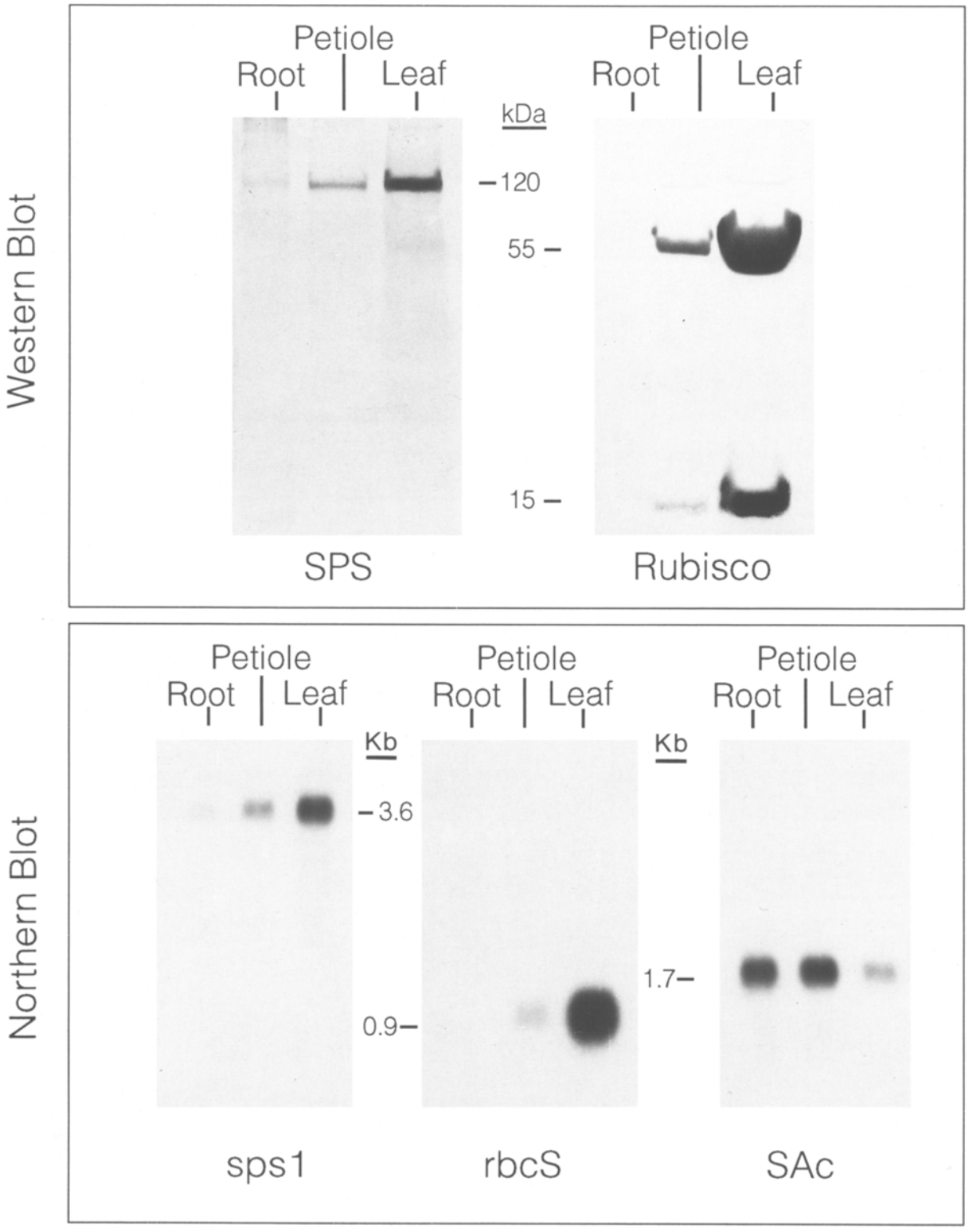


Fig. 4. Organ-specific accumulation of SPS protein and mRNA. Protein and poly(A)mRNA were extracted from petioles, leaves and fibrous roots of field-grown spinach. Western blots (*upper panel*) were loaded on an equal-protein basis (20 μg protein per lane) and were probed with antibodies to spinach SPS or tobacco Rubisco holoenzyme. Northern blots (*lower panel*) were loaded on an equal-poly(A)mRNA basis (0.5 μg mRNA per lane) and were probed with radiolabeled antisense RNA to SPS (*sps*1), Rubisco small subunit (*rbcS*) or Actin (*SAc*). Northern blots of *sps*1 *rbcS* and *SAc* RNA were exposed to x-ray film for 72 h, 1.5 h, and 24 h, respectively

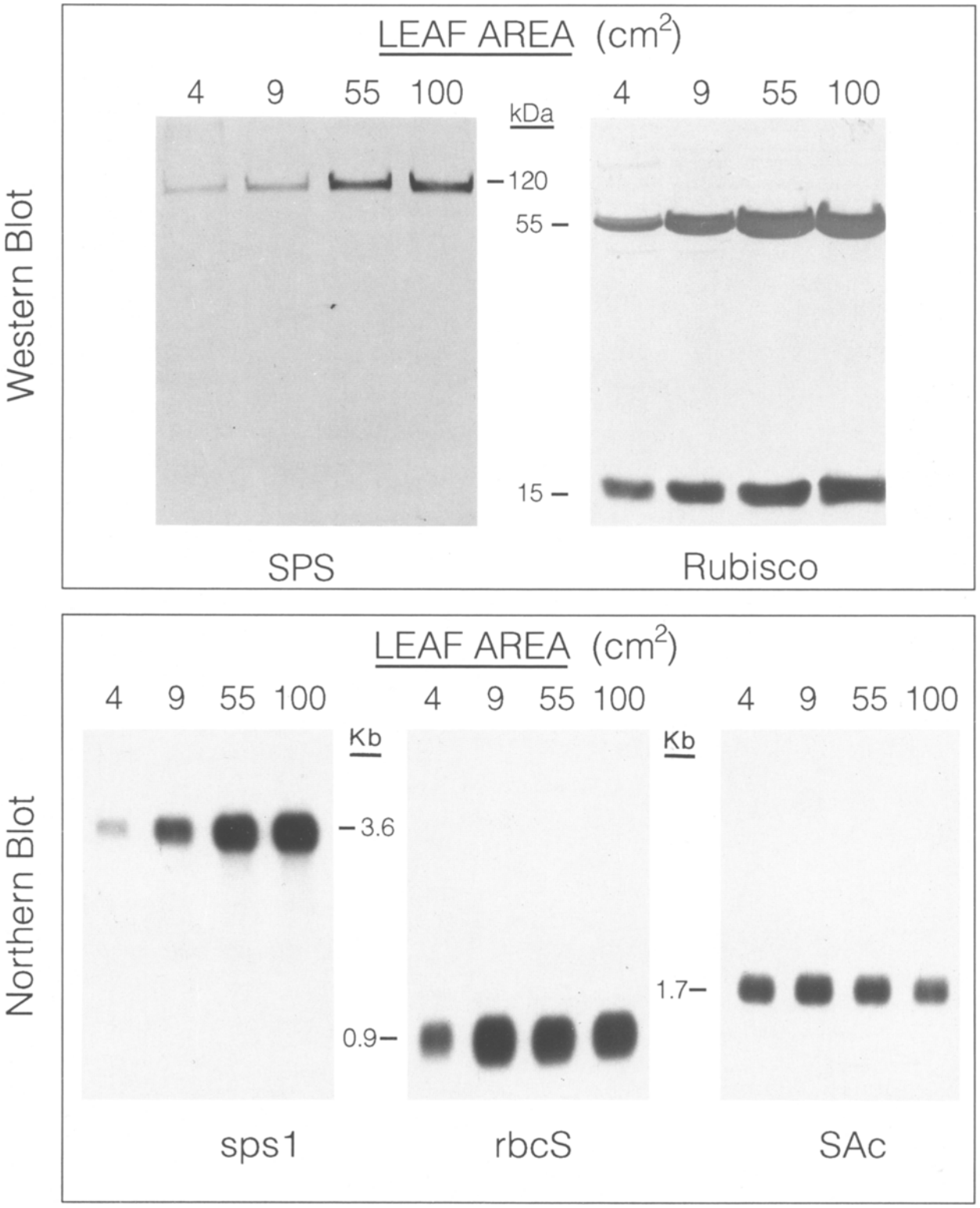


Fig. 5. Accumulation of SPS protein and mRNA during leaf development of spinach. Field-grown spinach leaves were harvested and separated into four size groups based on leaf area. Leaves of approx. 4, 9, 55, or 100 cm^2 were grouped and SPS protein and mRNA quantified. Western and Northern analyses were conducted as described for Fig. 4. Northern blots of *sps1*, *rbcS* and *SAc* mRNA were exposed to x-ray film for 8 h, 0.3 h, and 2 h, respectively

To determine whether altering the photosynthetic environment modulates SPS gene expression, young, rapidly expanding leaves (leaf area 5 cm^2) of chamber-grown spinach were shaded (photon fluence rate of 10 µmol · m^{-} $2 \cdot s^{-1}$) and were allowed to develop under these low-irradiance conditions (Fig. 6). Development of leaves in low irradiance resulted in a characteristic shade morphology typified by a reduction in leaf size and a reduction in the

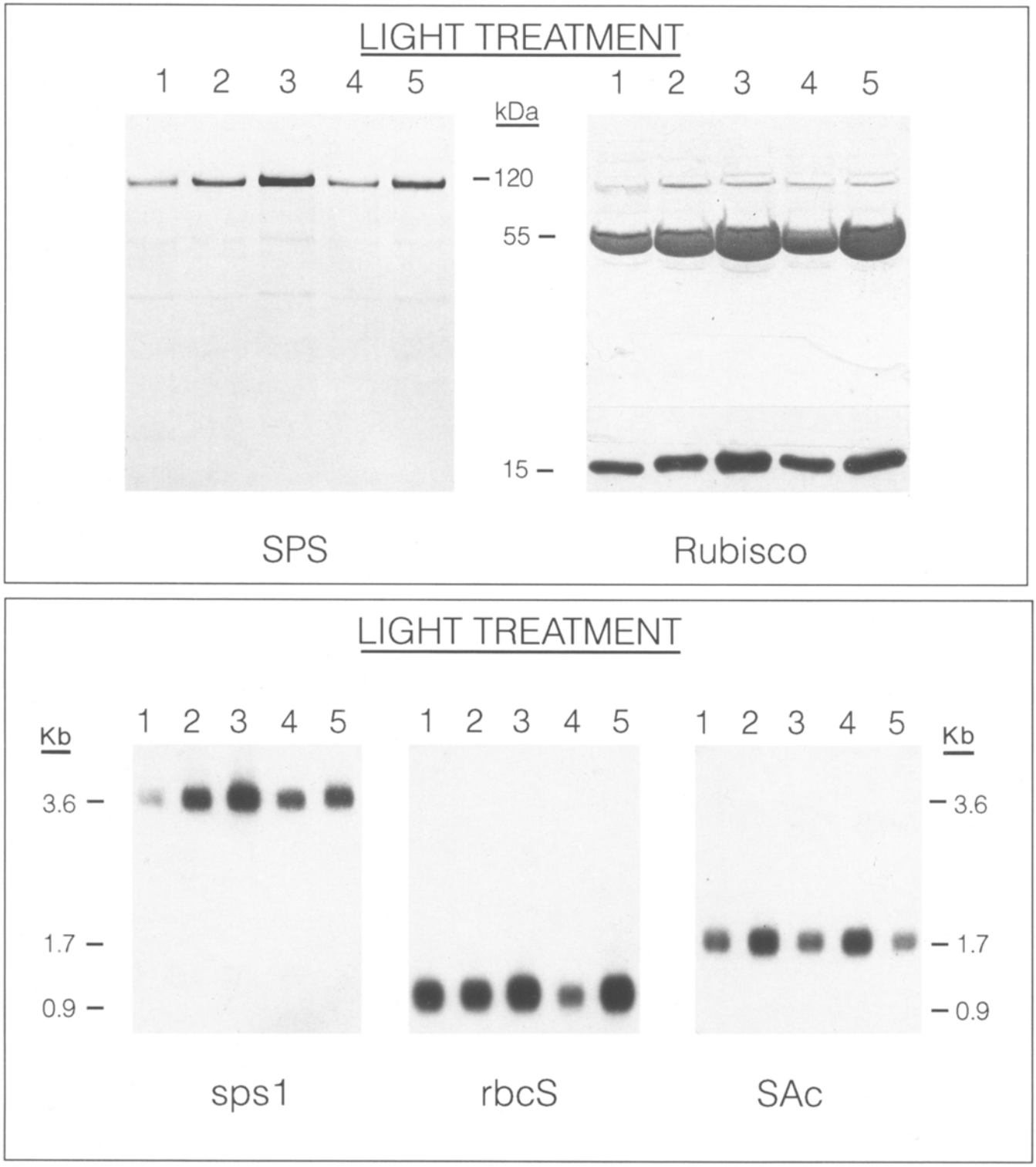


Fig. 6. Effect of irradiance during leaf development on SPS protein and mRNA accumulation. *Lane 1*, chamber-grown spinach leaves with leaf area of approx. 5 cm^2 (T_0 point); *lane 2*, T_0 plus 12 d shade ($\downarrow$10 $\mu mol \cdot m^{-2} \cdot s^{-1}$); *lane 3*, T_0 plus 12 d light (300 $\mu mol \cdot m^{-2} \cdot s^{-1}$); *lane 4*, T_0 plus 19 d shade; *lane 5*, T_0 plus 12 d shade plus 7 d light. Western and Northern analyses were subsequently conducted as described for Fig. 4. Northern blots of *sps*1, *rbcS*, and *SAc* were exposed to x-ray film for 14 h, 0.7 h, and 5 h, respectively

levels of Rubisco protein and transcripts (compare lanes 2 vs. 3; 4 vs. 5). Leaves that developed in low irradiance also exhibited reduced levels of SPS protein and mRNA when compared with nonshaded leaves (lanes 2 vs. 3). If the shade treatment was subsequently removed, a gradual adaptation to higher irradiances (350 $\mu mol \cdot m^{-2} \cdot s^{-1}$) was typified by an increase in leaf expansion and an increase in Rubisco protein and *rbcS*-transcript levels

Table 2. The effect of low irradiance during leaf development on SPS activity

Treatment of leaf[a]	SPS activity (μmol sucrose·h^{-1}·(g FW)$^{-1}$)	
	Limiting assay	V_{max}
12 d shade	50.0±20.4	112.7±7.3
12 d light	63.4±10.6	200.9±8.7
19 d shade	33.9± 5.7	96.6±7.8
12 d shade + 14 d light	65.5± 9.1	206.8±6.8

[a] Leaves were shaded (10 μmol·m^{-2}·s^{-1} light) or were placed in lighter conditions (300 μmol·m^{-2}·s^{-1} light) for the indicated durations

(lanes 4 vs. 5). Compared with leaves maintained in low irradiance, levels of SPS protein and transcripts were greater after shaded leaves were transferred to higher-irradiance conditions for 7 d (lane 4 vs. 5). Fourteen days after transfer of shaded leaves to higher-irradiance conditions, levels of SPS transcripts and protein equaled those of light-grown leaves (data not shown). Quantitation of SPS activity showed lower activity in shaded leaves than in leaves exposed to higher-irradiance conditions (Table 2). Differences in SPS activity were most apparent when assayed under substrate-saturated (v_{max}) conditions. In contrast, actin (*SAc*)-transcript levels showed a unique pattern of accumulation during leaf development in low-irradiance conditions. The accumulation of actin mRNA was substantially greater in leaves developing under shaded conditions (Fig. 6, lanes 2 vs. 3). In fact, when shaded leaves were returned to illuminated conditions, actin mRNA levels declined over the 7-d period while levels in shaded leaves remained high (compare lanes 4 vs. 5).

To investigate whether diurnal fluctuations in light intensity modulate SPS gene expression, leaves of field-grown spinach were harvested at various times throughout the photoperiod and SPS protein, mRNA, and enzyme activities were quantified. When SPS enzyme activity was assayed under saturated substrate conditions (V_{max}), no diurnal rhythm in enzyme activity was observed. A maximum extractable activity of approx. 215 μmol sucrose · (g FW)$^{-1}$ · h^{-1} was measured throughout the photoperiod. Similarly, a diurnal rhythm of SPS gene expression was not observed since the levels of SPS protein and mRNA remained relatively constant throughout the photoperiod (Fig. 7). By comparison, a slight diurnal rhythm in *rbcS*-transcript accumulation was detected but no apparent diurnal rhythm in actin-transcript accumulation was observed. The levels of *rbcS* transcripts reached an apparent maximum by 10 a.m. which is in general agreement with previous reports of a diurnal rhythm in *rbcS* gene expression (Piechulla 1988). The only apparent effect of light–dark transitions on SPS was restricted to a modulation of enzyme activity when assayed under conditions of limited substrate (data not shown). Under these enzyme assay conditions, a two-fold difference in SPS enzyme activity was apparent between midday (12 noon) and the activity at the end of the light period (8 p.m.).

Discussion

The present study detailed a PCR-based cloning strategy for isolating a full-length cDNA of the spinach SPS gene, utilizing partial amino-acid sequence information of the enzyme. The low abundance of the enzyme in spinach tissue prompted the use of PCR since the screening of a cDNA library with degenerate oligonucleotide primers would require screening large numbers of clones under low-stringency conditions. Further, the low abundance of the SPS enzyme made it difficult to produce a high-titer, specific antibody with which to screen a cDNA library. The PCR-based cloning strategy featured relatively long (26–31 mers) degenerate primers with highly reduced complexity and a template of random-hexamer-primed first-strand cDNA. The PCR amplification of a 561-bp product of the SPS gene facilitated the rapid screening of a bacteriophage library under stringent conditions and provided the necessary nucleotide sequence to utilize PCR-RACE in cloning a full-length cDNA. Hence, a homologous molecular probe of the spinach SPS gene was obtained thereby permitting the examination of SPS gene expression.

Regulation of SPS gene expression. Many plant genes are expressed in a highly regulated manner. Gene products may be present only in certain cell types, at specific stages of development or only following the application of distinct environmental stimuli (Kuhlemeier et al. 1987). The present results indicated that SPS gene expression is regulated in an organ-specific manner at the level of transcription or RNA stability. Examination of transcript and protein levels revealed that SPS gene products predominately accumulate in photosynthetic leaf tissue, though a smaller, but detectable, portion was also observed in petioles and roots. The organ-specific expression of SPS was similar to that observed for *rbcS* although *rbcS* transcripts and protein could not be detected in root tissue. It is well documented that the abundance of transcripts for *rbcS* increases markedly following exposure to light, particularly in cells that contain chloroplasts (Kuhlemeier et al. 1987, 1989). The involvement of chloroplasts in producing photoassimilate utilized in sucrose biosynthesis may explain the predominance of SPS in photosynthetic leaf tissue. However, it should be stated that SPS activity has also been observed in the scutellum of rice (Nomura and Akazawa 1973), in etiolated bean cotyledons (Brown and Huber 1987), and in developing fruits (Dali et al. 1992; Hubbard et al. 1989). While these tissues are largely nonphotosynthetic, they are involved in the biosynthesis and mobilization of stored reserves as sucrose. Further studies are necessary to elucidate the metabolic and environmental signals governing tissue- and organ-specific expression of SPS.

Examination of SPS transcript and protein levels during spinach leaf development revealed that the activation

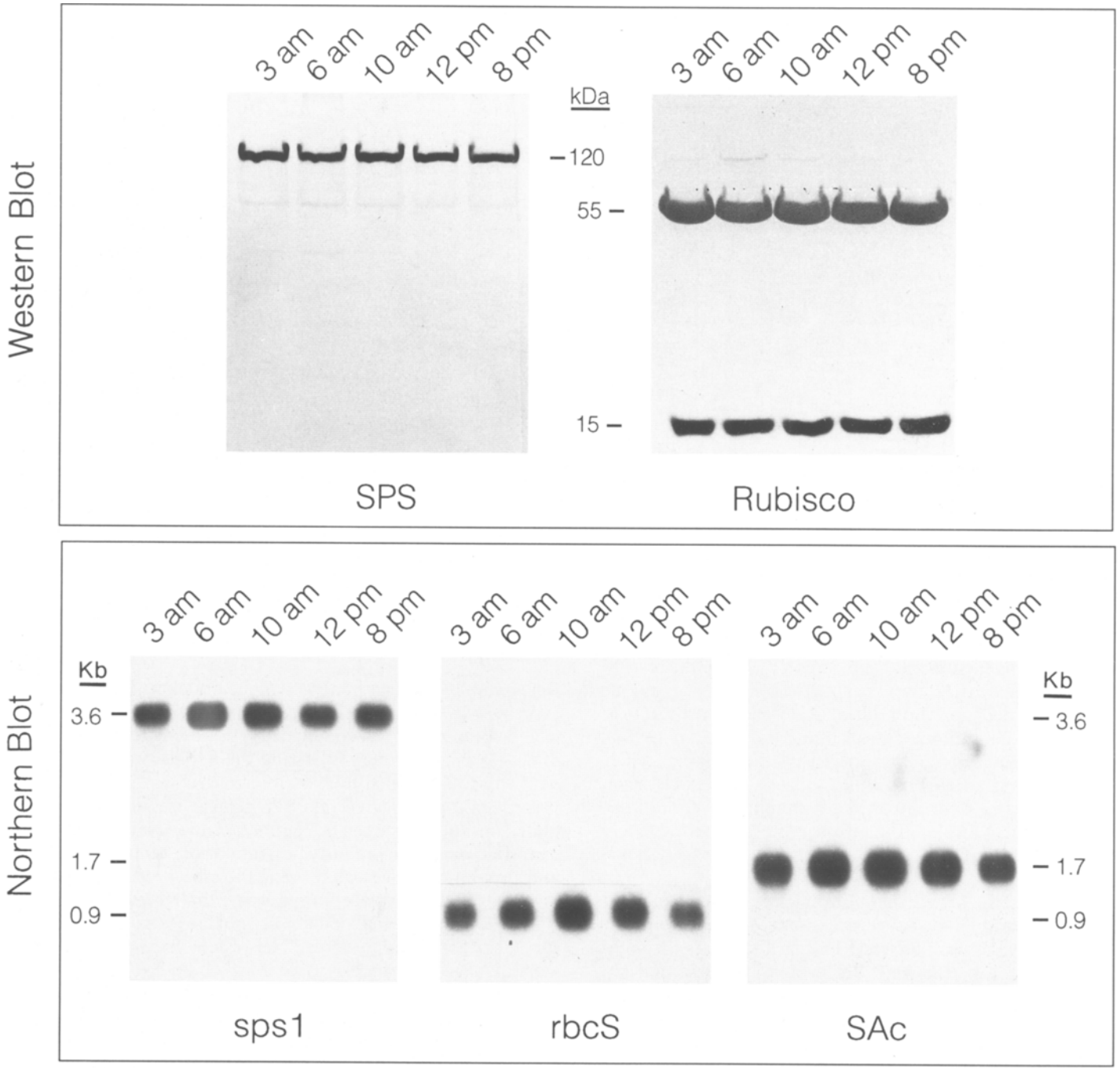


Fig. 7. Examination of the diurnal pattern of SPS protein and mRNA accumulation in spinach leaf tissue. Leaf tissue was collected from field-grown spinach at the time points depicted. Sunrise occurred at 6:15 a.m. and sunset at 7:30 p.m. Western and Northern blot analyses were conducted as described for Fig. 4. Northern blots of *sps*1, *rbcS*, and *SAc* were exposed to x-ray film for 12 h, 0.5 h, and 4 h, respectively

of SPS gene expression coincides with the apparent transition from a sink to a source leaf and the associated increase in photoassimilate export capacity of the leaf. In dicotyledonous plants, the transition from photoassimilate sink to source status begins shortly after the leaf has begun to unfold (for review, see Turgeon 1989; Silvius et al. 1978). Leaves of dicotyledons stop importing and begin to export when they are 30–60% fully expanded. Import appears to decline considerably while the leaf is still actively growing with import slowing after the rate of growth has peaked. The developmental pattern of SPS transcript and protein accumulation in spinach leaves coincides well with the apparent photosynthetic status of dicotyledonous leaves. Transcript and protein levels of spinach SPS increased during the period of rapid leaf expansion while no further accumulation was apparent after leaves had reached approximately half-full expansion. Hence, an unknown developmental signal associat-

ed with the transition in leaf carbon status regulates SPS gene expression and regulation is exerted at the level of gene transcription or transcript stability.

The present results indicated that the adaptation of leaves to either low or moderate irradiances can regulate SPS gene expression. The modulation of SPS transcript and protein accumulation during adaptation to low- or moderate-irradiance conditions paralleled the accumulation of Rubisco protein and *rbcS* transcripts. Previous studies of light acclimation during leaf expansion have shown that the irradiance under which a leaf develops affect the capacity of a number of physiological processes of photosynthesis, including leaf chlorophyll content, size of photosynthetic unit, chloroplast ultrastructure and a reduction in the Rubisco content of the leaf (Bowes et al. 1972; Bunce et al. 1977; Silvius et al. 1979). The concomitant decrease in SPS and Rubisco leaf content in shade leaves and the recovery of SPS and Rubisco leaf content upon exposure to higher irradiances may indicate a causal relationship between the SPS gene regulation and photosynthetic activity of the leaf. However, several pleiotropic effects of low irradiance on leaf ultrastructure were also observed, including a reduction in leaf size and specific leaf weight. An alteration in cellular ultrastructure under shaded conditions is also evidenced by the change in actin gene expression. Hence, the present study can not establish whether the photosynthetic capacity of the leaf or other unidentified developmental parameters control SPS gene expression during leaf expansion.

In many plant species, including spinach, a rapid alteration of leaf SPS activity occurs during dark-to-light transitions (for review, see Stitt et al. 1987). An additional level of control of many light-regulated enzymes exists at the level of gene expression (for review, see Kuhlemeier et al. 1987). Diurnal regulation of gene expression is indicated in the present study by the modest diurnal pattern of *rbcS*-transcript accumulation. However, it is apparent from the present analysis that SPS gene expression is not light-activated since transcript and protein levels remained relatively constant throughout the photoperiod. Immunochemical studies by Walker and Huber (1989b) and Bruneau et. al (1991) have also demonstrated that the change in SPS enzyme activity is not associated with a change in protein level. Hence, the response of SPS activity to light/dark transitions is strictly modulated by the activity of existing enzyme, although the mechanism apparently varies among species. On the basis of studies with spinach leaf SPS, Huber and Huber (1990) have proposed that protein phosphorylation is the mechanism responsible for light activation. With the isolation of genes encoding SPS from maize and spinach, it should now be feasible to identify amino-acid residues which are involved in light-dependent covalent modification and to identify those modifications that may confer species specificity.

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